



# Evaluation of Non-destructive Molecular Diagnostics for the Detection of *Neoparamoeba perurans*

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Amoebic gill disease (AGD) caused by *Neoparamoeba perurans*, has emerged in Europe as a significant problem for the Atlantic salmon farming industry. Gross gill score is the most widely used and practical method for determining AGD severity on farms and informing management decisions on disease mitigation strategies. As molecular diagnosis of AGD remains a high priority for much of the international salmon farming industry, there is a need to evaluate the suitability of currently available molecular assays in conjunction with the most appropriate non-destructive sampling methodology. The aims of this study were to assess a non-destructive sampling methodology (gill swabs) and to compare a range of currently available real-time polymerase chain-reaction (PCR) assays for the detection of *N. perurans*. Furthermore a comparison of the non-destructive molecular diagnostics with traditional screening methods of gill scoring and histopathology was also undertaken. The study found that all molecular protocols assessed performed well in cases of clinical AGD with high gill scores. A TaqMan based assay (protocol 1) was the optimal assay based on a range of parameters including % positive samples from a field trial performed on fish with gill scores ranging from 0 to 5. A higher proportion of gill swab samples tested positive by all protocols than gill filament biopsies and there was a strong correlation between gill swabs tested by protocol 1 and gross gill score and histology scores. Screening for *N. perurans* using protocol 1 in conjunction with non-destructive gill swab samples was shown to give the best results.

**Keywords:** Atlantic salmon, amoebic gill disease, *Neoparamoeba perurans*, molecular diagnostics, method validation

## INTRODUCTION

Amoebic gill disease (AGD), caused by *Neoparamoeba perurans*, is a major health challenge for the global Atlantic salmon (*Salmo salar*) farming industry (Rodger, 2014; Oldham et al., 2016). AGD has affected the marine Atlantic salmon industry in Tasmania since the 1980's and has since been described in farmed salmon in Ireland (Rodger and McArdle, 1996), Norway (Steinum et al., 2008),

Chile (Bustos et al., 2011) as well as France, Scotland and the Faroe Islands (Rodger, 2014; Oldham et al., 2016). In addition to Atlantic salmon, AGD has also been described in a number of other marine fish species (Oldham et al., 2016) including cleaner fish species used as a biological control of sea lice in Atlantic salmon farms (Haugland et al., in press).

If left untreated, AGD can cause significant mortality, up to 10% of livestock per week (Munday et al., 2001). The economic cost of a challenging issue is often the catalyst with regards to prioritizing research and the management of resources (Costello, 2009). Current AGD management practices are resource demanding and labor intensive, involving numerous bath treatments throughout a production cycle. Freshwater bathing has been the standard method of treating the disease in Tasmania but is limited by access to freshwater (Nowak et al., 2014). In cooler production areas, hydrogen peroxide is an effective treatment, but the treatment is recognized as having a narrow safety margin at higher temperatures (Adams et al., 2012) or where fish are compromised by advanced AGD (McCarthy et al., 2015). Some estimates have put the cost of AGD-related mortality between \$12.55 million in Norway and \$81 million in Scotland (Shinn et al., 2015).

The case definition for AGD is through histopathology, where amoebae are observed with associated pathology (Clark and Nowak, 1999; Rodger, 2014). By far the most widely used and practical method for ascertaining AGD severity and hence triggers for intervention (freshwater bathing, hydrogen peroxide treatments) is the gross gill score across all 16 hemibranchs, as described by Taylor et al. (2009), which may be coupled with histopathology and fresh microscopy to confirm the presence of lesion-associated amoebae. The identification of *N. perurans* as the causal agent of AGD (Young et al., 2007) has allowed the development of specific DNA based molecular diagnostic assays for the detection of the amoeba. Currently there are two conventional polymerase chain-reaction (PCR) assays published for the detection of *N. perurans*, (Young et al., 2008; Rozas et al., 2011), while three real-time PCR assays were developed based on SYBR® green (Bridle et al., 2010) and TaqMan® chemistries (Fringuelli et al., 2012; Downes et al., 2015).

A standardized molecular diagnostic method has the potential to fulfill a role as an early warning and monitoring tool which would greatly complement traditional diagnostic methods, particularly in the early stages of infection when gross clinical signs may be absent and in other fish species for which the gill scoring method is less applicable. The aims of this study were to compare two non-destructive methods of sampling for *N. perurans* to confirm AGD, gill swabs and gill filament biopsy samples taken from the same animal during a naturally occurring infection in a field trial. A range of currently available molecular assays for the detection of *N. perurans* were compared with regards to sensitivity, specificity and practicality, utilizing the samples taken from the field trial. Furthermore, the preferred molecular assay used to test gill swab samples was then compared with the traditional screening methods of gross gill scoring and histopathology, on samples taken during an experimental infection trial.

## MATERIALS AND METHODS

### Field Trial

The field trial consisted of commercial all-female diploid Atlantic salmon which were put to sea as smolts in south east Tasmania, Australia, on the 8th of July 2014. The samples were collected on the 13th of March 2015 when the fish were an average weight of 1.5 kg. All fish had previously been subjected to 5 freshwater bath treatments, the last of which was on 25th of February 2015. At the time of sampling, biomass in the cage was 96,407 kg (5.7 kg m<sup>-3</sup>). Five fish were selectively sampled from each gill score (scores 0–5 assessed across all gill surfaces, Taylor et al., 2009) ( $n = 30$ ) with individual scores being recorded for each fish. The second gill arch on either side of each fish was used for tissue sampling, a gill filament biopsy was conducted on the right side targeting  $25 \pm 2$  mg per sample while the front and back of the left side second arch was swabbed with isohelix swabs (Cell Project Ltd.). Both the filament and swab samples were stored in 2 mL screw-cap micro-centrifuge tubes in 100% AR ethanol for transport and storage.

### Sample Preparation and DNA Extraction

Swab samples were placed into tissue lyser (Qiagen) for 10 min at a frequency setting of 20.0 Hz before vortexing and pulse centrifuging of each individual tube. The swabs were removed using a sterile forceps taking care not to cross-contaminate samples. Both swab and filament samples were then spun down at 21,130 g for 10 min in order to form a visible pellet for swab samples and to facilitate ethanol removal. Extraction was then completed using the DNA easy mini kit (Qiagen) according to manufacturer's instructions.

### Real-Time PCR (qPCR) Evaluation and Protocols

The published methodologies available at the time for evaluation are listed in Table 1. Qualitative analysis of each assay was assessed in relation to the percentage of positive results for the gill swabs and filament samples in the field trial. The sensitivity, specificity, linearity and correlation to gill score of each assay were also analyzed. In order to investigate the sensitivity of the assays, a plasmid was created and its concentration determined as previously described (Downes et al., 2015). A dilution series was generated and analyzed by the three real-time assays (protocols 1, 2, and 4) to assess the lowest copy numbers detectable. Only dilutions that produced *Ct*-values in all triplicates were included in the analysis. DNA extracted from *in vitro* cultures of *N. perurans* obtained from three countries Norway, Ireland and Australia were used to assess the specificity of each of the assays. Additionally, the assays were appraised with respect to cost (in AUS\$) per sample (cost of reagents for each assay for a single sample run in triplicate) and time requirements (runtime for each method in relation to through-put). All results were reviewed and each assay was then ranked on a scale of 1 to 5, with 1 being the most optimal score. These scores were combined and in order to compare the assays.

For each protocol, primers were obtained from Sigma (for work completed in Ireland) or GeneWorks (for work completed

**TABLE 1 | A list of the real-time PCR protocols evaluated in this study in addition to the salmon elongation factor assay used as an external process control.**

Protocol	Gene	Target	Primer/ Probe	Name	Sequence (5'–3')	Product Size (bp)	Reference
1	18S rRNA	N. perurans	Forward	NP1	AAAAGACCATGCGATTGTAAGT	70	Downes et al., 2015
			Reverse	NP2	CATTCTTTTCGGAGAGTGGAAATT		
			Probe	NPP	6-FAM- ATCATGATTACCATATGTT-MGB		
2	18S rRNA	N. perurans	Forward	Peru.F	GTTCTTTTCGGAGCTGGGAG	139	Fringuelli et al., 2012
			Reverse	Peru.R	GAAGTATCGCCGGCAGAAAAG		
			Probe	Peru.P	6-FAM-CAATGCCATTCTTTTCGGA-MGB		
4	18S rRNA	N. perurans	Forward	QNperF3	GTTTACATATTATGACCCACT	146	Bridle et al., 2010
			Reverse	QNperR3	TAAACCCAATAGGTCTGC		
5	18S rRNA	N. perurans	Forward	Nper.F	ATCTTGACTGGTTCTTTTCGGGA	636	Young et al., 2008
			Reverse	Nper.R	ATAGGTCTGCTTATCACTYATTCT		
External Process Control	ELF	ELF Salmonid	Forward	S-ELF.F	GGCCAGATCTCCCAGGGCTAT	66	Bruno et al., 2007
			Reverse	S-ELF.R	TGAAGTTGCAGGCGATGTGA		
			Probe	S-ELF.P	6-FAM-CCTGTGCTGGATTGCCATACTG-MGB		

Protocol 3 is commercially available as a kit and does not include information on the primer/probe sequences.

in Australia). TaqMan probes and master mix for protocols 1 and 2 were purchased from Life Technologies.

## Protocol 1

Protocol 1 is a TaqMan<sup>®</sup> qPCR targeting the 18S rRNA gene sequence of *N. perurans* generating an amplicon of 70 bp (Downes et al., 2015).

## Protocol 2

Protocol 2 is a TaqMan<sup>®</sup> qPCR targeting the 18S rRNA gene sequence of *N. perurans* generating an amplicon of 139 bp (Fringuelli et al., 2012).

## Protocol 3

Protocol 3 was a commercial kit developed by Primerdesign Ltd for the detection of *N. perurans*. Each reaction mixture and thermal profile was completed following manufacturer's instructions.

## Protocol 4

Protocol 4 was a SYBR<sup>®</sup> Green protocol targeting the 18S rRNA gene sequence of *N. perurans* generating an amplicon of 146 bp (Bridle et al., 2010). Each qPCR reaction contained 0.4  $\mu$ M of each primer, 2x SensiFAST SYBR<sup>®</sup> Lo-ROX Master Mix (Bioline) and nuclease-free water. Following optimization of this protocol (unpublished) it was determined that addition of neat DNA was not appropriate, resulting in  $C_t < 10$ , therefore samples were diluted to  $\sim 5$  ng  $\mu$ l<sup>-1</sup> and 2  $\mu$ l (10 ng) of DNA was added to each reaction.

The reaction was incubated at 95°C for 10 min followed by 45 cycles of 95°C for 10 s, 60°C for 15 s and 72°C for 34 s. Following the 45 cycles a melt curve analysis was performed to determine the specificity of the reaction. A 5-point standard curve based on

a known quantity of cultured cells (chosen from 100, 50, 25, 10, 5, and 1 cell and also diluted down to 5 ng  $\mu$ l<sup>-1</sup>) was included in each run. To validate the results the melting temperature of the qPCR products were compared with the  $T_m$  of the culture ( $T_m \sim 77^\circ\text{C}$ ). A sample was considered positive if the  $T_m$  was between 75 and 77.5°C.

## Protocol 5

Protocol 5 was a modified nested PCR based on the first round amplification described by (Young et al., 2008) which amplifies a 636 bp region of the *N. perurans* 18S rRNA gene followed by a 1:5 dilution of the PCR product in nuclease-free water and analyzing further using protocol 4. The nested PCR consisted of 0.365  $\mu$ M of each primer, 2x GoTaq<sup>®</sup> Colorless Master Mix (Promega) and nuclease-free water. This initial amplification was completed for all samples, the full range of the known cell standard curve (100, 50, 25, 10, 5, and 1 cell) and also a Nested No Template Control (Nested NTC). As in Protocol 4, samples and the standard curve were diluted to 5 ng  $\mu$ l<sup>-1</sup> and 2  $\mu$ l (10 ng) of DNA added.

For each protocol, all samples were run in triplicate on an Applied Biosystems AB7500 Real-Time instrument and associated software. Each run included a positive control, a negative control and a non-process control. An external process (salmonid elongation factor-1 $\alpha$ ; Bruno et al., 2007) control was used for each sample.

## Amoebae Culture

In order to conduct an infection trial for the comparison of the preferred assay with traditional screening methods, *N. perurans* was isolated from farmed Atlantic salmon affected by AGD in the west of Ireland using a method described in Downes et al. (2015), adapted from Morrison et al. (2004). The amoeba culture was established and maintained according to Crosbie et al. (2012).

To confirm the presence of *N. perurans* in the culture, a sub-sample of the culture was tested by conventional PCR (Young et al., 2008). Amoebae were harvested by physical removal from the agar using a bacteriological spreading bar, followed by several seawater washes. The amoeba seawater solution was then collected in a sterile flask. Several counts of the amoeba seawater solution were undertaken using a 1 ml Sedgewick Rafter Counting Chamber (SPI Supplies).

## Infection Trial

The infection trial was carried out at the Daithi O'Murichu Marine Research Station, Bantry, Co. Cork, Ireland using four 400 L flow-through tanks at full salinity which were each stocked with 50 Atlantic salmon smolts weighing ~70 g. Following an acclimation period of 2 weeks, two of the tanks were challenged with cultured *N. perurans* and two other tanks were used as negative controls. For the infected tanks, the water level was lowered and inoculated with amoeba at 1,000 cells L<sup>-1</sup> for 4 h. Throughout the trial the fish were fed 1% of body weight per day, water quality was monitored daily and the temperature was constant at 11–12°C throughout the trial. This work was authorized by the Health Products Regulatory Authority (HPRA) in Ireland under project authorisation number AE19114/P001, following the Animals Scientific Procedures Act 1986 (Directive 2010/63/EU transposed into Irish law by S.I. No 543 of 2012).

Sampling commenced 24 h post-infection with further samples taken at 2, 3, 8, 15, and 21 d post-infection. At each sampling point, three fish were sampled from each tank. Gross gill scoring of individual fish ( $n = 3$ ) was conducted onsite using the gill scoring system described by Taylor et al. (2009). The second gill arch on the right-hand side was swabbed (Isohelix) and processed as described above for the field trial. Gill swab samples were tested by real-time PCR (Protocol 1). The second gill arch on the left-hand side was excised from each fish and immediately fixed in 10% neutral buffered formalin for histological processing. Sections (5 µm) from paraffin embedded tissue were stained with haematoxylin and eosin and examined microscopically on an Olympus BX51 microscope. In order to determine if there was a correlation between gross pathology and histopathology, a scoring system based on that described by Mitchell et al. (2012) was applied to determine the progression and severity of gill lesions in fish where AGD developed. A score of 0 = normal gill; 1 = low pathology <10% of gill tissue affected; 2 = moderate pathology <50% of gill tissue affected and 3 = severe pathology > than 50% of gill tissue affected. Only histological sections where pathology was observed in the presence of amoeba were recorded as AGD infected (Clark and Nowak, 1999; Rodger, 2014).

## Statistical Analysis

Regression analysis was carried out in order to determine the lowest detectable copy number of each of the assays. Spearman's correlation analysis was undertaken to assess the relationship between gross gill pathology scores, histopathology scores and PCR analysis (Minitab 17). Kappa statistics were conducted in order to determine the level of agreement between PCR analysis, gill scoring and histopathology.

## RESULTS

### Field Trial

Polymerase chain-reaction (PCR) results of each of the five protocols for both gill swabs and filaments are shown in **Figure 1**. Overall, more positive results were detected by each protocol for gill swab samples compared with gill filament biopsies. Protocol 1 gave 100% positive gill swabs, down to 55% with protocol 4. Protocol 1 gave 79% positive results with gill filament biopsy, compared with 14% for protocols 3 and 4.

The percentage positive results for each protocol, for both gill swab and gill filament biopsy, across each gross gill score (0–5) are shown in **Table 2**. A higher number of positive results were found when using gill swabs rather than filament biopsies. There is a general increase of positive swab samples with increasing gross gill score, all protocols had 100% positive results at gill score 5 whereas only protocols 1 and 3 gave 100% positive results at gill scores 0–3. In relation to the percentage of positive filament biopsy samples, only protocol 1 found 100% of samples positive at gill scores of 2, 4, and 5.

### Linearity, Efficiency, Sensitivity, and Specificity

The known numbers of cells examined was 25, 10 and 1 amoebae cells and were analyzed by protocols 1, 2, 4, and 5 only. Each assay performed well with multiple cells, only protocols 1 and 2 were found to reliably detect *N. perurans* down to a single cell (**Table 3**). Both protocol 1 and 2 provided very similar results for the known quantity of cells and were shown to perform best when analyzing a single cell with average *Ct*-values of 35.10 and 35.51, respectively. These two assays also had similar linearity  $R^2$ -values of 0.9679 (Protocol 1) and 0.9605 (Protocol 2), while protocol 5 had the lowest  $R^2$ -value of 0.8171 (**Table 3**). Three of the assays were analyzed in relation to the lowest detectable copy numbers, the LOD determined for protocol 1 was 2.64 copies, protocol 2 was 14.7 and protocol 4 was 115 (**Table 4**). DNA extracted from cultures of *N. perurans* provided from three countries (Ireland, Norway and Australia) were analyzed to determine specificity and each protocol was found to perform comparatively (all results were positive). The amplification efficiency for each of the assays were found to be comparable and within the expected range of  $Ex = 90$ –110%.

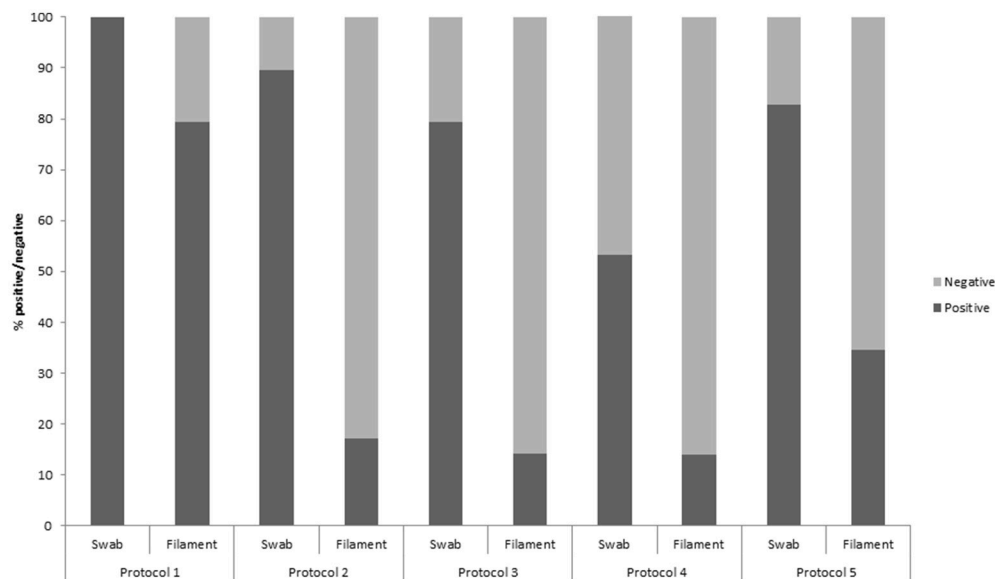
### Swab/Gill Score Correlation

There was a significant ( $P < 0.01$ ) negative correlation for protocols 1, 2, 4, and 5 when analyzing the *Ct*-values from the swab samples with the individual gross gill scores of the fish in the field trial (**Table 4**). The correlation between gross gill scores and gill filament samples was found to be significant for protocols 1, 2, and 5 ( $P < 0.01$ ), however, the correlation coefficient for each of the assays was noticeably lower for the gill filament samples when compared with gill swab samples (**Table 4**).

### Cost and Time Analysis

In relation to cost per sample, protocol 4 was the cheapest assay at \$2.76 AUD and protocol 3 the most expensive at \$24.38 AUD





**FIGURE 1 |** The percentage of gill swab and gill filament biopsy samples which were positive/negative for *Neoparamoeba perurans* during a natural outbreak of amoebic gill diseases in Atlantic salmon during a field trial, assessed by five qPCR protocols.

(Table 4). With respect to the time required based on a full 96-well plate, protocols 1, 2, and 3 take the same amount of time for analysis at 2 h 55 min, while protocol 5 took 6 h 20 min.

## Ranking

All of the results recorded for each of the parameters examined were compared and ranked from 1 to 5 (1 being the optimal) (Table 4). It was found that protocol 1 performed better for several of the parameters, such as PCR efficiency for known cell numbers, % of positive samples for swabs and filaments, % difference between the positive results for swabs and filaments, in addition to having the greatest correlation between swabs and gross gill score. Protocol 5 was ranked 1 for correlation in relation to the filaments and the gill score. Protocol 4 was ranked 1 in relation to cost.

## Infection Trial: Comparison of Gill Swab, Histology and Gross Gill Score

Results from the infection trial are shown in Figure 2. Using protocol 1, the first PCR positive samples were detected 2 d post-infection (16%) and by 15 d post-infection 100% of the samples were positive. Both gill score and histology scores first increased above score 0 on 8 d post-infection and continued to increase throughout the remainder of the trial. Gross pathology, as characterized by white mucoid spots and plaques on the gill surface, was first recorded 8 d post-infection. Amoebae were first observed during histological examination 15 d post infection. There was a significant correlation ( $P > 0.01$ ) between each of the methods analyzed (Table 5). There was a significantly negative correlation between the PCR results and both the gill score ( $-0.938$ ) and, the histology score ( $-0.836$ ). Conversely, analysis between the gill score

and histology score expressed a significant strong positive correlation (0.849). There was excellent concordance between the PCR and gill score ( $K = 0.80$ ) and between gill score and histology score ( $K = 0.80$ ). *N. perurans* DNA was detected in all samples where AGD was microscopically diagnosed, while overall agreement between the PCR and histology score was good ( $K = 0.69$ ).

## DISCUSSION

The identification of *N. perurans* as the causative agent of AGD (Young et al., 2007; Crosbie et al., 2012) has allowed the development of a range of molecular based diagnostic assays for the detection of the amoeba. While it is acknowledged that alternative molecular diagnostic assay are available (Haugland et al., in press; Hellebø et al., in press) they have not been published in the literature and therefore could not be included in this study. Therefore, this study has compared a range of published molecular assays available at the time and has shown that gill swab samples are more sensitive than gill filament biopsies, resulting in higher number of positive results from known infected fish. Molecular based diagnostic methods were also shown to correlate well with the more traditional diagnostic methods of gill and histology scoring.

Regular gross gill scoring provides fish farmers with immediate information on AGD prevalence and intensity to support husbandry and treatment decisions. This method is particularly suitable on Atlantic salmon farms that are constantly affected by the disease, allowing operators to become familiar with the gross presentation of lesions. However, the gross gill score can be difficult to interpret when non-AGD pathologies, such as proliferative gill disease or gill necrosis are present

**TABLE 2 |** The percentage positive results for each protocol for both gill swab and gill filament biopsy samples in relation to gross gill scores in Atlantic salmon during a field trial undergoing natural outbreaks of amoebic gill disease.

Gross gill score	Swab					Filament				
	Protocol 1	Protocol 2	Protocol 3	Protocol 4	Protocol 5	Protocol 1	Protocol 2	Protocol 3	Protocol 4	Protocol 5
0	100	80	100	20	80	80	0	0	0	0
1	100	80	100	60	60	40	0	20	0	0
2	100	80	100	20	60	100	20	20	0	20
3	100	100	40	60	100	60	0	20	0	40
4	100	100	80	80	100	100	60	20	60	80
5	100	100	100	100	100	100	40	0	40	80

**TABLE 3 |** Ct-values for known numbers of *N. perurans* cells and linearity assessed for protocols 1, 2, 4, and 5 (values are mean  $\pm$  SD).

	R <sup>2</sup> -value	Known no. of cells		
		25	10	1
Protocol 1	0.9679	31.26 $\pm$ 0.28	32.37 $\pm$ 0.15	35.10 $\pm$ 0.75
Protocol 2	0.9605	31.29 $\pm$ 0.26	32.35 $\pm$ 0.27	35.51 $\pm$ 0.51
Protocol 4	0.8885	30.89 $\pm$ 0.1	31.24 $\pm$ 0.49	36.43 $\pm$ 0.17*
Protocol 5	0.8171	26.05 $\pm$ 0.17	27.27 $\pm$ 0.25	39.39 $\pm$ 1.05*

\*Ct-values above the stated limit of detection for these assays.

(Steinum et al., 2010; Mitchell and Rodger, 2011). It is also reported that the gross gill scoring method is less applicable for other fish species affected by AGD, such as lumpfish (*Cyclopterus lumpus*) which are used as cleaner fish in Atlantic salmon cages (Haugland et al., in press).

It is clear from the results of this study that gill swabs improved the sensitivity in comparison to gill filament biopsies. Results collected from each of the PCR protocols demonstrated an increase in the number of positive samples detected when samples were taken using the swabs. In addition to the increase in positive detections, there was also a higher correlation between gill swab and gross gill scores across the majority of the assays. The difference observed between the two sampling methods (swabs v filament) is likely due to the greater gill surface area sampled by the swab and there may be a greater abundance of amoeba in the mucous collected on the swab than between the distal filaments. However, one parameter that is likely to have had an effect on the difference of sensitivity between the two sampling methods is that of the matrix effect where the presence of inhibitors affect the sensitivity of the assays (Schrader et al., 2012). There was an observed reduction in the amount of salmon DNA between the gill filament biopsy and the gill swabs when tested using the salmon ELF assay (data not shown). It must be noted that non-detection with some of the assays may be due to the quantity of amoebae DNA below the detection threshold of the assay rather than a technical failure of the assay (Collins et al., 2016).

The qualitative analysis of each protocol demonstrated differences between the positive/negative results produced. As the fish sampled during the field trial were taken from a naturally infected population, which was in its sixth round of AGD

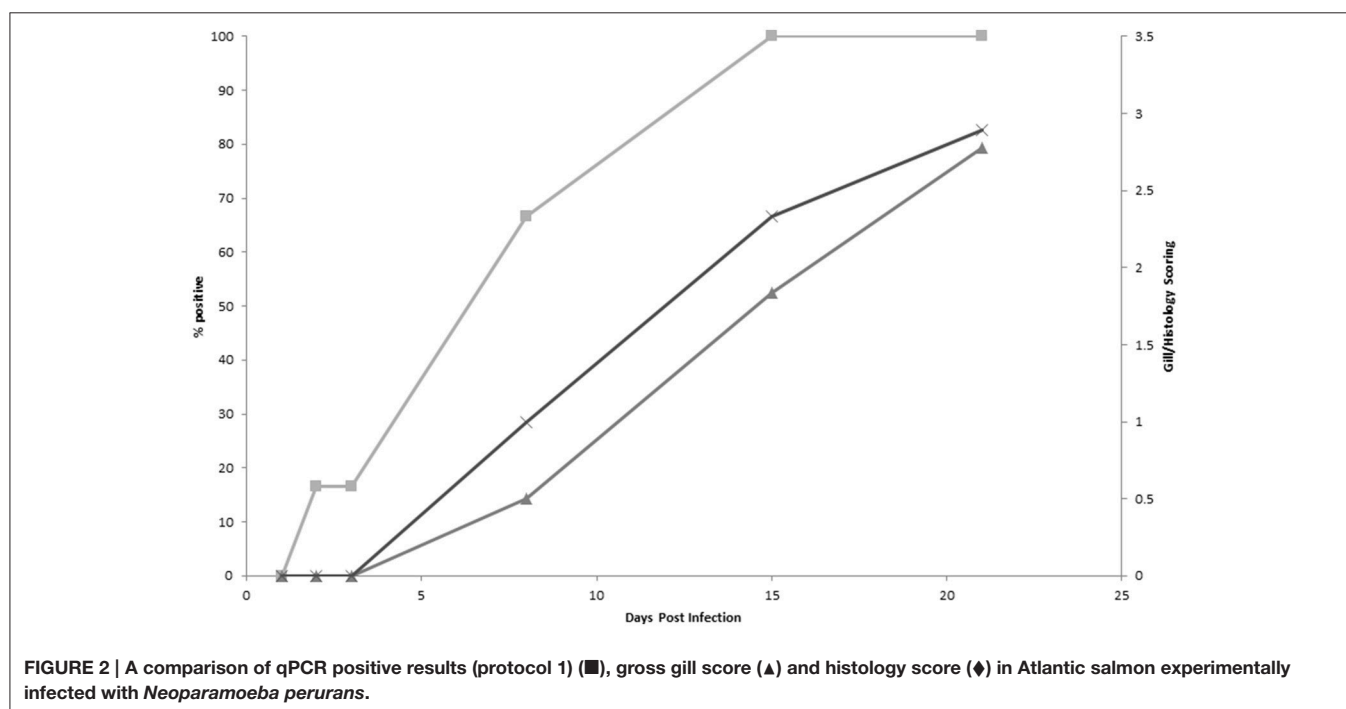
infection, it enabled the study to sample a broad range of AGD gross gill scores. The Taqman assays (protocols 1 and 2) produced relatively similar results with respect to the swabs. Conversely, there was a stark difference between the results produced for the filament samples using these two protocols. A shorter amplification fragment and with primers designed closer to the probe in protocol 1 appears to have increased the sensitivity of the assay thus ensuring more positive results when testing the filament samples. The effect of a smaller amplicon size has previously been found to be advantageous for TaqMan assays in the presence of inhibiting compounds (Opel et al., 2010).

When using protocol 4 the melt curve analysis produced multiple peaks, which suggests a composite of more than one product with melting temperatures considerably different to that of the standard/positive control. Samples that produced Ct-values, but a product with a different melting temperature were deemed to be negative. When analyzing the samples with protocol 5 (which was a modified protocol 4), no additional peaks were found for the swab samples and only 20% of the filament biopsy samples produced additional peaks in the melt curve analysis suggesting that the nested PCR (protocol 5) appears to increase the specificity of the SYBR assay (protocol 4). Multiple peaks found in melt curves of SYBR green analysis is generally indicative of non-specific amplification as SYBR green is a dye that binds to all double-stranded DNA molecules, the specificity of an assay based on this chemistry is due only to the choice of primers (Martenot et al., 2010). Consequently, mispriming and the formation of primer dimers can produce false positive results in addition to overestimates of DNA quantities (Bustin, 2000). In other comparisons of SYBR green and TaqMan chemistries where there are low copy numbers of the gene, there is a greater accumulation of primer dimers and non-specific double stranded DNA by the SYBR Green Chemistry (Hein et al., 2001; Martenot et al., 2010). Additionally, TaqMan<sup>®</sup> chemistry is generally thought to offer a number of advantages over SYBR<sup>®</sup> green, in particular, the incorporation of minor-groove-binders (MGB) which allow for the raising of melting temperatures of the probes (enabling the use of shorter probes) and integration of the internal hydrolysis probe providing greater specificity in comparison to the intercalating dye assays, which have reduced specificity because any amplification product incorporates the dye. (Gunson et al., 2006; Purcell et al., 2011).

**TABLE 4 | Ranking of the real-time PCR protocols on a range of parameters assessed by analyzing samples of naturally infected Atlantic salmon in a field trial.**

Protocol	Qualitative analysis			Sensitivity			Correlation to gill score		Additional factors	
	% +ve swabs	% +ve Filaments	% difference swabs vs. filaments	Linearity of assay ( $R^2$ )	DNA copy no. (cells $\mu\text{l}^{-1}$ )	Swabs ( $R^2$ )	Filaments ( $R^2$ )	Cost per sample (AUD\$)	Time (h:min)	Overall ranked by median
1	100	79	21	0.9679*	2.64	-0.689*	-0.608*	\$6.50	2:55	<b>1.0</b>
2	90	17	82	0.9605*	14.3	-0.666*	-0.48*	\$7.36	2:55	<b>2.5</b>
3	79	14	82	N/A	N/A	-0.109	-0.069	\$24.38	2:55	<b>4.0</b>
4	55	14	75	0.8885*	115	-0.656*	-0.445*	\$2.76	4:45	<b>3.5</b>
5	83	34	59	0.8171*	N/A	-0.606*	-0.618*	\$3.18	6:20	<b>3.0</b>

\* $P < 0.05$ . Values in bold are the overall median rank for each of the assays following the review of the results for each of the parameters assessed.

**TABLE 5 | Spearmans correlation between gill swab qPCR results, histology score and gross gill scores in Atlantic salmon experimentally infected with *Neoparamoeba perurans*.**

	Gill score	PCR
qPCR	-0.938	
Histology scores	0.849	-0.836

Each assay was designed to amplify specific regions of the 18S rRNA gene, which is generally chosen due to its high copy number, thus potentially increasing sensitivity. The 18S rRNA gene is an established marker for microbial identification, used in numerous studies, with a large database of species specific sequences (Bridle et al., 2010). Another reason for this choice is that multiple copies of this gene are encoded within the eukaryotic genome (Long and Dawid, 1980; Young et al., 2008). Sensitivity in relation to the lowest detectable DNA copy numbers was assessed for protocols 1, 2, and 4. Two of the protocols

were not assessed for DNA copy numbers due to availability of information on the primer sequences for protocol 3 and for protocol 5 which was based on the Young et al. (2008) primers which were used to produce the plasmid DNA. From the analysis of the three protocols assessed it was found that protocol 1 was able to detect the lowest concentration of copies of DNA at 2.64 copies  $\mu\text{l}^{-1}$ , which approaches the theoretical limit of detection (Purcell et al., 2011). Analysis of protocol 2 gave a concentration of 14.3 copies  $\mu\text{l}^{-1}$  which is similar to levels reported by Fringuelli et al. (2012). Protocol 4 has previously been described as having an LOD of 1.418 copies  $\mu\text{l}^{-1}$ , however this was not achievable during this study and was found to be able to detect 115 copies  $\mu\text{l}^{-1}$ . Further dilutions produced multiple peaks and incorrect melting temperatures, making melt curve analysis difficult.

Correlations between the molecular results and the gill scores were consistent for each of the protocols used during the field trial for both gill swabs and filament biopsies. Previously Bridle et al. (2010) reported excellent agreement between AGD gross

gill scores (Powell et al., 2001) and molecular results (gill scores 1 through to 4, no gill score 0), though the gill area sampled, fish size and season are not specified. The results of the current field study showed lower agreement, which may reflect the wider gill score range used in this study (from 0 to 5). The samples were taken in early Autumn (Australia), which is typically associated with a slowing of AGD pathology and the onset of non-specific gill necrosis prior to winter. A higher correlation between gill score and gross pathology was recorded in the naïve smolt during the infection trial. This may be an indication of the differences between initial and subsequent infections (as the fish in the field trial had undergone previous cycles of AGD and freshwater treatment) and may also reflect differences in host tolerance or resistance to amoeba exposure between naïve smolts and larger fish following several rounds of bathing and reinfection.

Taking into account the additional factors assessed for each of the assays, it is clear that the SYBR green protocols are generally cheaper to run as they do not require the inclusion of costly hydrolysis probes. The higher cost of the commercial product is due to the inclusion in the cost of several other reagents in the kit, required for controls and standard curves. In relation to time, the SYBR green assays require longer run times due to addition of melt curve analyses which result in a reduced throughput rate in comparison to that of the TaqMan protocols. Protocol 1 was seen to perform most favorably across most of the attributes assessed and was ranked by median score as the most suitable assay. Following this it was decided to compare protocol 1 with traditional screening methods (gross gill scoring and histopathology) during an infection trial.

The gross gill scoring, histological scoring and in particular the molecular data presented showed that detection of *N. perurans* was possible within 2 weeks post-infection and has been previously reported (Morrison et al., 2004; Taylor et al., 2007). Histological examination of the samples in this study identified pathological changes within the first week; however the observation of amoeba in the presence of pathology and therefore case definition was not confirmed by histology until the second week. While histopathology can indicate both the

presence of the pathogen and resultant host response, it requires destructive sampling which could potentially limit the scale of epidemiological studies (Douglas-Helders et al., 2001; Adams et al., 2004) and is not suitable for screening valuable fish e.g., tagged individuals in a breeding program. Molecular analysis did however confirm the presence of *N. perurans* in the first 48 h of infection and subsequently at each further sampling point. This clearly indicates the usefulness of this non-destructive molecular diagnostic assay for the early detection of *N. perurans* and in sub-clinical cases of AGD. Additionally, with significant gains made through selective breeding (as measured by reduced gill score, Kube et al., 2012) there is an opportunity to fully optimize non-destructive sampling techniques in conjunction with molecular methods to help inform management decisions, such as when to treat fish and also determine the efficacy of treatments, and to more finely measure potential gains in AGD resistance/resilience.

## AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: JD, RT, MC, EM, and NR. Performed the experiments and sample analysis: JD, MR, RT, BM, MM, and EC. Writing and editing manuscript: JD, RT, EM, IO, HR, NR, and MC.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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